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Progress in enzyme immunoassays: production of reagents, experimental design, and interpretation*

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Enzyme immunoassays represent in many cases the preferred procedure for the detection of antigens or corresponding antibodies. However, many of the current procedures are performed suboptimally. This article reviews the available designs, auxiliary recognition systems, production and purification of antibodies, conjugation procedures, solid-phase materials, recording and interpretation of results, and quality control and standardization of procedures to improve the reproducibility of tests.

INTRODUCTION

Enzyme immunoassays (EIA) and enzyme immunohistochemistry (EIH) have supplanted or expanded many of the traditional techniques in diagnostic medicine and biological research. These procedures, which are simple to perform and give excellent results, take advantage of two important biological phenomena: the potentially very high specificity of antibodies for a given antigen and the extremely powerful amplification of chemical reactions achieved with enzymes. All such procedures consist essentially of two steps, i.e., the immunological reaction and the enzymatic indicator reaction to demonstrate the presence or absence of antibody-antigen reactions. Problems involving specificity may arise, however, if the starting antiserum has undesired reactivities.

Several factors have had a major impact on the development of these assays. The labelling procedure of immunoreactants with enzyme has been refined so that conjugates with high immunological and enzymatic activities can be obtained. Secondly, antigens and antibodies can be fixed efficiently to solid-phase materials. Thus, the antigens need not be produced in the cells to be detected, and they are generally present in higher concentrations than would be possible in cells. Moreover, the antigens that cannot be produced in cells, as well as antibodies, can be immobilized. Thirdly, assays can be designed with high detectabilities, or high sensitivities. Other advantages are that relatively cheap equipment is required, the assays are feasible in field conditions and may be performed rapidly and easily, and the health hazards are limited. In addition, the development of the hybridoma technique for the production of monoclonal antibodies has given an extra boost to the use of EIA.

Although many different systems have been reported for diagnostic or research purposes, the present review will concentrate on the principles, the basic techniques, and the pitfalls. Many reagents for EIA and EIH have become commercially available and the prices reflect a considerable commercial overhead; the former poor quality, however, has been significantly improved in recent years.

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OVERVIEW OF EIA DESIGNS

A plethora of enzyme immunoassays has been reported and given rise to a confusing terminology. However, all of them are based on one of two fundamentally different systems called Activity Amplification (AA) assays and Activity Modulation (AM) assays. Most immunoassays employed are of the AA type.

The theoretical limit of the number of molecules that can be detected by the AA method is 1. This has not been achieved in practice (the limit is still about 10^4 molecules), although Rotman (*81*) succeeded in measuring the activity of single β-galactosidase molecules. In the AM assays, the limit is given by the ratio of the experimental error to the affinity (avidity) constant, and these assays therefore have up to about 10^6 lower detectability than AA assays. On the other hand, the specificity of the AA methods is considerably less than that of AM methods since, in the former, the cross-reacting antigens appear equipotent owing to the excess of immunoreactants. However, the use of monoclonal antibodies and sandwich methods (use of two different antisera) may produce a specificity bonus in the AA methods.

Noncompetitive, solid-phase enzyme immunoassays by the AA method can be divided into two groups according to the immunoreactant immobilized on the solid phase.

For the detection of antibodies generally, the antigens that trap antibodies (if present in the sera to be tested) are immobilized and they can be detected by enzyme-conjugated anti-immunoglobulin antibodies. The detectability of this method can be improved by the use of bridge methods, in which the captured antibody is detected by nonimmunologic (avidin-biotin, protein A) or immunologic ("a-ELISA" (*11*, *93*)) methods. An exception to these general designs is the class capture assay used to distinguish IgM, IgG or other immunoglobulin classes. In these assays, the antibody and not the antigen, e.g., anti-IgM or anti-IgG antibody, is immobilized on the solid phase which serves to capture the IgM or IgG from the test sera. Subsequent incubations with antigen, antibody, and conjugate serve then to reveal the presence of IgM or IgG antibodies and their activity. The EIH methods, which may detect both antigens and antibodies, are fundamentally similar to this group of assays (*53*, *55-58*).

The second group of enzyme immunoassays of the AA type comprises the noncompetitive, homogeneous methods. These do not require a solid phase or separation in stages (*71*). Two monoclonal antibodies with specificities to neighbouring, but different, epitopes are labelled with selected enzymes so that one produces the substrate for the other (e.g., glucose oxidase and peroxidase).

Competitive, homogeneous enzyme immunoassays are seldom used in the diagnosis of infectious diseases, in contrast to the competitive, solid-phase EIA. In the latter method two approaches can be distinguished. In the first, antibody is immobilized on the solid phase and the corresponding antigen (from test or standardization solutions) has to compete with a standard amount of enzyme-labelled antigen for the available binding sites. A more important decrease in staining indicates a larger amount of antigen in the test solution. Alternatively, antigen may be immobilized which will bind a certain amount of enzyme-labelled antibody. If, however, free antigen is present in the test sample, it may compete for this labelled antibody and thus decrease the response. The decrease with standard amounts yields the standard curve which serves to measure the amount of antigen present in the test samples.

For the detection of antigens, the antibodies that capture antigens from the test samples are immobilized, and the antigens are detected by the methods described above. However, care should be taken in these sandwich methods to include controls to verify whether the antibodies subsequently used to detect the antigen do not react with the antibody immobilized on the solid phase.

AUXILIARY RECOGNITION SYSTEMS IN EIA

Molecular recognition forms the basis not only of enzyme immunoassays but also of all living systems. Among these nonimmunologic recognition systems, all can theoretically be employed for EIA, but a few are employed with great success in these assays (avidin-biotin, protein A, and lectins).

Avidin-biotin

Avidin has an exceptionally high affinity for *d*-biotin (10^{15} mol^{-1}) and has 4 binding sites for biotin. A maximum of two of these sites can be used since the occupation of one binding site creates a steric hindrance for another. However, avidin can act as a very effective bridging molecule between two biotin moieties. Biotin is a small molecule with a carboxyl group at the end not involved in binding. This carboxyl group may be activated to render biotin reactive towards proteins (enzymes, antibodies, or antigens). Green (*32*) has reviewed this system in detail. Avidin is isolated from egg white; a similar molecule, streptavidin, is isolated from *Streptomyces* and seems to have a lower background staining in EIA.

Streptavidin is commercially available[a] but the cost is very high. Avidin is widely available and a cheap and simple procedure to obtain avidin directly from homogenized egg whites (yield about 1 mg/egg white) has been reported by Heney & Orr (*41*). Homogenized egg white (3-fold dilution with water) is brought to 70% saturation with ammonium sulfate. The supernatant obtained after centrifugation is brought to 100% saturation, left overnight, and the pellet obtained after centrifugation is dissolved in water (1.5 ml per egg) and dialysed 3 times against 100 ml of water (per egg). The pH is adjusted to 11 with 1 mol/l NaOH and 2 mol/l NaCl is added. A small column of 2-iminobiotin-6-aminobensyl-agarose, equilibrated with 0.05 mol/l sodium carbonate and 1 mol/l NaCl, is used to adsorb the avidin from the extract. After the absorbance at 282 nm has returned to the baseline, the addition of 0.05 mol/l ammonium acetate, pH 4.0, will elute the pure avidin.

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Two types of activated biotin are most commonly used, biotinyl-*p*-nitrophenyl (PNB) ester and biotinyl-*N*-hydroxysuccinimide (BNHS) ester. Both are commercially available (e.g., Sigma). We generally prefer BNHS, because it is much more water-soluble and seems to contain less harmful by-products. However, BNHS should be stored dry to prevent hydrolysis.

Biotinylation of proteins is quite simple. The number of amino groups in the proteins should be taken into account as well as the optimum ratio of biotin ester (the amino group is quite narrow for most satisfactory EIAs). Guesdon et al. (*33*) showed that biotinylation affected the enzymatic activities of glucose oxidase and peroxidase minimally, of β-galactosidase moderately, and of alkaline phosphatase to a considerable degree. The antigen-binding capacity of the antibodies does not seem to be affected. For biotinylation, the proteins should be present at a concentration of at least 10 mg/ml (in 0.1 mol/l carbonate buffer, pH 8.5). The PNB or BNHS is dissolved just before use in dimethylformamide or dimethylsulfoxide to obtain a concentration of 0.1 mol/l. Either of these esters is then mixed with the protein solution in a w/w ratio of 1:6 (peroxidase) or 1:3 (IgG) of biotin ester and protein.

Protein A

Another frequently used reagent in enzyme immunoassays is protein A (isolated from *Staphylococcus aureus*, usually the Cowan I strain). This protein has the interesting property of reacting with many immunoglobulins from mammalian species (not from birds). Protein A thus resembles an anti-immunoglobulin with, however, the additional advantage that it may react with antibodies of different species and, since it has two binding sites, act as a bridging molecule between immunoglobulins of different species. Moreover, enzyme-labelled protein A can be employed as a general tracer. The properties of this protein have been reviewed recently by Langone (*60*).

Protein A binds immunoglobulins primarily through the Fc site, though reactions with the Fab site have been reported. The association constant of protein A for rabbit IgG is around 10^8 mol^{-1} but may vary strongly for IgG or its subclasses from other species. An interesting phenomenon is that binding of the antigen at the Fab site enhances the affinity between the Fc portion of the IgG and the protein, probably owing to antigen-induced aggregation (*82*). The reaction is not affected by Tween-20, Triton X-100 or Brij-97 at the concentrations used in EIA or EIH.

A major disadvantage of protein A is the high cost, but crude extracts of this product are satisfactory for most enzyme immunoassays. Protein A may be produced in the laboratory according to the method of Sjöquist et al. (*85*). A stirred fermenter with a working volume of 10 litres will yield about 200–250 g of bacteria, from which about 400 mg of protein A can be isolated. The major expense is the 10 mg of lysotaphin (from Sigma) which is needed during the purification procedure. The method to obtain the crude extract (i.e., by ammonium sulfate precipitation) is quite simple. To further purify the protein A, ion-exchange chromatography and gel filtration are commonly used.

Lectins

Lectins often mimic the action of antibodies in their affinity for specific carbohydrates. These lectins, some of which have been named agglutinins or phytohaemagglutinins, have found wide application in biochemistry, serology and oncology. They have been extensively reviewed by Gold & Balding (*29*) and Goldstein & Hayes (*30*).

In general, lectins are extracted from finely ground seed meal, though their source is not restricted to plants. The classifications of lectins are not systematic, but most attempt to take into account their specificity for the sugar groups they recognize (*30*).

PRODUCTION AND PURIFICATION OF ANTIBODIES

Immunization

An understanding of the principles of the immune response is an essential prerequisite for the successful production of antibodies. This topic is, however, outside the scope of this review. Though immunization is largely empirical, some general characteristics should be stressed.

For the production of polyclonal antisera, utmost care should be devoted to the purification of the immunogen. Extra time invested at this stage is nearly always well spent. Traces of very immunogenic impurities may overwhelm the response to the principle immunogen (immunogen is defined as the substance that induces the immune response, whereas antigen is the substance which is able to react immunologically with the antibody). For the production of monoclonal antibodies the purity of the immunogen is not a prime prerequisite.

A second requirement for the production of high-titred antisera is that the immunogen should be recognized as foreign, i.e., the animal should not have been in contact with the virus or other immunogen prior to immunization. It is, therefore, always necessary to obtain sera from the animals before immunization to establish their immune status.

Several refinements are possible in order to increase the specificity of the antibodies produced. Proteins purified on polyacrylamide gels may, after staining with Coomassie Brilliant Blue (electrophoretic destaining should be avoided), be used directly as immunogens, the gel acting as an adjuvant (*8*). Another elegant method is first to raise an oligospecific antiserum against an immunogen in the presence of contaminants. This antiserum can then be used to generate an immunoprecipitate with the antigen in agarose, e.g., with crossed immunoelectrophoresis. These immunoprecipitates can be cut carefully from the gel and used, after washing in neutral saline, for the immunization of a second animal (*9*) to obtain monospecific antisera. This method requires two succeeding immunizations, but may be an excellent choice if the first immunization was less successful or if it is difficult to remove the contaminants.

Immunization procedures vary from laboratory to laboratory. Most often rabbits are used; however, their genetic constitution is generally not known. For inbred mice it has been shown that both the antibody titre obtainable and the avidity are genetically determined. Harboe & Ingild (*38*) observed considerable differences in antibody titres in 17 outbred rabbits immunized with horseradish peroxidase. These variations should be considered and it may often be useful to immunize several animals. A poor responder will not increase significantly during prolonged immunization.

Adjuvants are commonly used to enhance humoral responses to weak immunogens. Most useful are incomplete Freund's adjuvants, alum (to precipitate proteins), and methylated bovine serum albumin (for negatively charged materials). The failure to make a proper emulsion of immunogen solution and Freund's adjuvant is a common cause of unsuccessful immunization. A simple method has been described by Herbert (*42*). The mixture can be stored in small aliquots at −20 °C, but should be shaken vigorously before each immunization.

Subcutaneous inoculations are convenient but act more slowly than intramuscular inoculations. Very little or nothing can be gained by injecting Freund's emulsions at shorter intervals than 1 month. The affinity generally increases with time and, with longer intervals, between boosts. The rabbits should be at least 3 months old and should be injected with about 25 μg of immunogen per kg in the thick part of the skin above the shoulder blade. Trial bleedings should be included. A simple method for repeated bleeding of rabbits is to use the central ear artery (*31*). In this method an 18-gauge hypodermic needle, without the hub, is inserted 5–10 mm into the central ear artery after the dorsal surface of the ear has been moistened with 70% alcohol and shaved, and the terminal 1 cm of the ear been wetted with xylene (upper and lower surface). Prior to inserting the needle, the ear is briskly massaged over the central artery, and on the first occasion 40 ml of blood is collected in a centrifuge tube; at subsequent bleedings (weekly intervals) the volume may be increased gradually to 200 ml.

A recent, increasingly popular method to obtain antisera for EIA is by immunization of hens. Bleeding is then not necessary since the egg yolk contains up to 15 mg of antibodies (IgY). Eggs thus provide the equivalent of more than 100 ml of serum per week. Purification of IgY is quite simple (*48*), but avian antibodies do have the disadvantage that they do not bind protein A.

Relative merits of polyclonal and monoclonal antibodies

Animals may produce several thousand clonotypes against a given epitope (*51*). It is, therefore, impossible to obtain antisera with similar properties from different animals. Even antisera taken successively from the same animal differ in their properties.

Monoclonal antibodies, on the other hand, are produced by a single clone which can be maintained *in vitro* over very long periods and can be used to generate large quantities of identical antibodies (e.g., in the ascitic fluid of mice). A uniform, specific, and constant affinity for a certain epitope is thus obtained. It should be stressed, however, that two important properties of polyclonal antisera that help to protect the animal, i.e., the high general affinity (avidity) and the specificity, are lost in the procedure of monoclonal antibody preparation. Theoretically, these can be regained by mixing the appropriate monoclonal antibody solutions (*21*). Enzyme immunoassays require a certain threshold in avidity which is often, however, not achieved with monoclonal antibodies.

Nevertheless, monoclonal antibodies offer new possibilities to simplify EIA and to design new assays. Examples of assays made possible by the availability of monclonal antibodies are the simultaneous two-site immunometric asay and the proximal linkage immunometric assays (*71*).

Standardization of reagents for EIA is an important advantage when using monoclonal antibodies, compared with polyclonal antibodies. Monoclonal antibody production has been described in numerous reviews (*23*, *28*, *45*, *78*, *95*).

Purification of immunoglobulins

About 10% of the antiserum proteins are immunoglobulins, of which usually 1–10% are antibodies to the injected immunogen. An important enrichment of antibodies can thus be achieved by the purification of immunoglobulins. Consequently, less enzyme will be needed to conjugate the same amount of antibodies and background staining in subsequent enzyme immunoassays will be substantially reduced.

The most general method of antibody purification is based on selective precipitation of ammonium or sodium sulfate. It should be stressed that the percentage values (in which their respective concentrations are expressed) differ between the two salts. Thus, for ammonium sulfate, 20% means that the solution contains 20% of the salt needed to saturate that solution, whereas for sodium sulfate this is given as the percentage of weight per volume. Thus 20% sodium sulfate refers to 20 g of this salt per 100 ml, whereas 20% ammonium sulfate means 0.8 mol/l (about 10.5 g/100 ml). Moreover, saturation is temperature-dependent, in particular for sodium sulfate.

Saturated ammonium sulfate solutions should be prepared carefully. The pH should be adjusted with diluted ammonium hydroxide and should not be measured directly in the solution (essentially "dry"), but in 20-times diluted aliquots from the main solution. Otherwise the error will exceed one pH unit. An important characteristic of ammonium sulfate is its density (1.245 g/ml). It is, therefore, important to add the salt solution very slowly under continuous mixing to avoid localized high concentrations leading to the precipitation of contaminants. A final concentration of 33% ammonium sulfate at 0 °C is usually most satisfactory. The mixture is equilibrated at 0 °C for one hour before centrifugation (for 30 min, 4000 *g*). The pellet is resuspended in a neutral isotonic buffer (same volume as originally) and the precipitation is repeated once (the pellet is then taken up in half the volume of buffer).

For large amounts of antisera the method of Harboe & Ingild (*38*) is very convenient. In 100 ml of antiserum, 25 g of ammonium sulfate is dissolved and left at room temperature for at least 3 hours. Centrifugation as above yields 98% of the antibody activity in the pellet. The supernatant is discarded, and the precipitate is washed with 25 ml of 1.75 mol/l (43%) ammonium sulfate, collected by centrifugation, and washed again.

The precipitate may contain lipoproteins which can be removed by the following method. The precipitate is taken up in a small amount of water, and dialysed at 4 °C for 12 hours against distilled water, for 24 hours against 0.05 mol/l sodium acetate and 0.021 mol/l acetic acid (pH 5.0), then twice for 12 hours against water and once for 24 hours against the acetate buffer. The precipitate (mainly lipoprotein) is then removed by centrifugation. The supernatant contains predominantly IgG and some IgA. A convenient method to check the degree of purification is by measuring the ratio of the optical densities at 278 and 251 nm. This ratio is at least 2.5 for IgG, whereas for the contaminating proteins the ratio is around 1.

Immunoglobulins can be further purified by ion-exchange chromatography. New ion-exchangers such as DEAE-Sephacel are particulary convenient. A simple method described by Levy & Sober (*63*) is commonly employed. Rabbit immunoglobulins are dialysed against 0.0175 mol/l phosphate buffer, pH 6.5, and passed through a pre-equilibrated DEAE-Sephacel column. All contaminants are retained whereas the IgG can be directly collected. A variant of this method, employed by Harboe & Ingild (*38*), is equally simple. About 1 g of DEAE-Sephadex A-50 is suspended in 50 ml of 0.025 mol/l sodium acetate and left overnight. The excess buffer is discarded and a column is packed with this gel (25 ml). The sample, in 0.05 mol/l sodium acetate and 0.021 mol/l acetic acid (pH 5.0), is applied and IgG (and IgA) will pass through directly. The same buffer is applied (elution speed 60 ml/h) and the eluate is monitored. Prior to storage, the antibodies are dialysed against 0.1 mol/l sodium chloride.

Affinity chromatography, in particular with protein A-Sepharose C1-4B, is rapidly becoming a popular tool for the separation of many different mammalian IgG and even their subclasses (*60*).

A simple method was reported by Goding (*28*). The IgG, e.g., from rabbit, is adsorbed to the protein A-Sepharose in the column. After all contaminants have passed, the IgG can be desorbed by elution with a 0.58% acetic acid solution containing 0.9% sodium chloride.

Purification of antibodies

Though the purification of antibodies by immunoaffinity chromatography seems ideal, it is quite often risky. The antibodies with the highest affinity, which are the ones determining the sensitivity of EIA, are frequently lost during these procedures or they may be denatured during the harsh treatment needed to dissociate the antigen-antibody complexes. However, immunosorbents may also be used to deplete a serum preparation from contaminants. Immunoaffinity columns can be prepared after periodate activation of commonly available matrices such as agarose (Sepharose) or cellulose (*25*), or after CNBr-activation (*64*), or with *N*-hydroxysuccinimide-derived agarose (*52*, *68*). The isolation of specific antibodies on immunosorbents cannot be achieved by a single uniform elution method owing to the variation in interaction between antigen and antibody (i.e., affinity differences). Interesting studies in this respect are those of Kristiansen (*96*) and O'Sullivan et al. (*75*).

Preparation of Fab fragments

The use of Fab fragments in EIA will generally lower the background reading or staining. Moreover, they may be particularly useful in indirect sandwich assays, in which the Fab fragments are immobilized on the solid phase. Anti-immunoglobulins used as the layer in such tests generally recognize only the Fc fragments of bound antibody and hence will not bind to the Fab fragments by cross-reaction. Moreover, they may offer advantages for EIH because of their small size. However, a major possible disadvantage is the loss of the high general affinity.

The various immunoglobulins and their subclasses differ significantly in their sensitivity to proteolytic digestion (*16*, *86*). Parham et al. (*76*) and Lamoyi & Nisonoff (*59*) reported the preparation of Fab fragments from mouse monoclonal IgG. Proteins were dialysed overnight against 0.1 mol/l sodium acetate at 4 °C. The pH was subsequently lowered to 4.2 by adding 2 mol/l acetic acid. Pepsin (twice crystallized) was then added to an amount of 60% of the immunoglobulin weight. The mixture was incubated at 37 °C and the digestion was stopped by raising the pH to 8 by adding Na0H. The incubation time was dependent on the IgG subclass: 12 hours for IgG1, 4 hours for IgG2a, and 15 min for IgG3. IgG2b was hydrolysed very rapidly and only 10% activity was obtained after 15 min, which was associated with IgG still undegraded.

CONJUGATION PROCEDURES

Many different enzymes have been used for enzyme immunoassays; among these, peroxidase, β-galactosidase, alkaline phosphatase, and glucose oxidase have been most frequently used in diagnostic medicine (*14*, *54*, *94*).

The most appropriate enzymes, in our experience, are peroxidase and β-galactosidase. Alkaline phosphatase is frequently used in plant research since, in plant material, peroxidase activity may be considerable. However, alkaline phosphatase has serious drawbacks: it is expensive and not sensitive (compared with peroxidase or β-galactosidase), and conjugation procedures to obtain alkaline phosphatase conjugates in a defined form are lacking.

Peroxidase has the best detectabilities with colorimetric measurements, whereas β-galactosidase has the best detectabilities with fluorimetric measurements (*47*).

Purification of peroxidase

Commercially produced enzymes are quite often expensive. Recently, a very simple method to purify peroxidase from crude extracts was described, which takes about one hour. The potential savings are considerable (*58*, *89*). For example, commercially purchased crude extract at a cost of US$ 200 will yield an amount of pure peroxidase costing about US$ 2500 (prices from Boehringer catalogue, 1984).

DEAE-Sepharose is equilibrated with 2.5 mmol/l sodium phosphate buffer, pH 8.0; crude peroxidase (ratio of optical densities at 403 and 275 nm (= RZ), about 1) is dissolved in the same buffer and applied to the DEAE-Sepharose column (up to 5 mg protein per ml gel). Pure peroxidase, isozyme C, passes directly whereas impurities and less active isozymes are retained. The RZ of this peroxidase is higher than the commercial pure peroxidase, i.e., about 3.3, and the enzyme has a higher specific activity. The column may be regenerated by washing with 0.25 mol/l phosphate buffer, pH 8.0.

Conjugation procedures: principles

The most satisfactory conjugation procedures should yield 100% conjugate of well-defined composition without inactivation of the macromolecules involved. Many different techniques have been described, few of which can be recommended. For example, the two-step glutaraldehyde procedure (*4*) is widely used for peroxidase conjugation but yields, at best, poor results (1-10% conjugation).

The best conjugation methods are, however, not universally applicable. For example, the periodate method is eminently suitable for peroxidase or glucose oxidase but not for β-galactosidase or alkaline phosphatase. On the other hand, *o*-phenylenedimaleimide is very suitable for β-galactosidase but not for peroxidase.

Fundamentally, three different conjugation methods can be distinguished: (i) chemical cross-linking, (ii) immunological cross-linking, and (iii) bridge methods (e.g., avidin-biotin). Sometimes two methods may be combined, as in the antibody-chimera method (*77*). In the latter, a monoclonal anti-peroxidase antibody is linked chemically to an anti-immunoglobulin antibody, followed by the binding of peroxidase through the Fab sites. This method thus circumvents the difficulties encountered when peroxidase is to be conjugated, since immunoglobulins can be cross-linked quite easily.

A most important, but largely ignored, factor in conjugation procedures is the law of mass action, which states that the rate of complex formation is proportional to the product of the reactant concentration. For example, if the macromolecules to be conjugated are present at 1 mg/ml instead of, e.g., 5 mg/ml, prolongation of the incubation period by 25 times would be required to obtain the same amount of conjugates.

A second problem is that the activated molecules will not be distributed evenly over the target molecules when they are brought into contact. In our laboratory we found that this distribution can be described by a binomial model and it demonstrates that, irrespective of the molar concentration ratio of enzyme:IgG, not more than about 37% of the IgG will be conjugated with one enzyme molecule. Increasing the enzyme concentration beyond this optimum will decrease the amount of free IgG but increase even more the number of IgG molecules with two or more enzyme molecules.

A third factor to be taken into account is the degree of activation. These macromolecules may act as bridges between two or more of the target molecules, and large polymers will arise if more than one active group is introduced per macromolecule. It is generally observed that large polymers cause high background staining levels.

In most instances a very definite optimum of activation is recommended, followed by a purification of the conjugate. It has been shown elsewhere (*58*, *88*) that even a small amount of free antibody will decrease considerably the sensitivity of the enzyme immunoassay.

Selected methods for conjugation

Periodate procedure (method A) for peroxidase or glucose oxidase. This method has been described by Wilson & Nakane (*92*) and is a modification of the original method introduced by Nakane & Kawaoi (*70*). The carbohydrate moiety of the enzyme is oxidized with meta-periodate to generate aldehyde groups. These aldehyde groups are allowed to form Schiff's bases with the amino groups of the antibodies, which are subsequently stabilized with sodium borohydride.

The procedure is uncomplicated and is carried out as follows:

(i) dissolve 4–8 mg peroxidase (Sigma type VI, Boeringer type I, or purified as described above) in 1 ml of distilled water;

(ii) prepare a fresh solution of 0.1 mol/l of sodium meta-periodate in distilled water and add 0.1 ml to the peroxidase solution; stir gently for 20 minutes at room temperature;

(iii) dialyse overnight at 4 °C against a large excess of 1.0 mmol/l of sodium acetate buffer, pH 4.4;

(iv) raise the pH of the sample to 9.0–9.5 by the addition of 0.2 mol/l of sodium bicarbonate buffer, pH 9.5;

(v) add activated peroxidase immediately to the immunoglobulin solution (10 mg IgG in 1 ml) dialysed against 20 mmol/l of carbonate buffer, pH 9.5, and stir gently for two hours at room temperature;

(vi) add 0.1 ml of freshly prepared sodium borohydride (4 mg/ml) in distilled water and leave for 2 hours at 4 °C;

(vii) dialyse the conjugated mixture against 0.1 mol/l of borate buffer, pH 7.4, overnight at 4 °C;

(viii) purify the conjugate on Sephadex G-200 or by the method given in the next section.

Optimized periodate procedure (method B). The central problem in the periodate method is the sensitivity of the carbohydrate moiety to oxidation (*58*). Too little oxidation will not activate the enzyme satisfactorily; however, excessive oxidation is often more serious. The aldehyde groups generated are more sensitive to oxidation than the original vicinal glycol groups. Thus, progressively more carboxyl groups appear, which are useless for conjugation. Peroxidase in particular is quite sensitive to oxidation, and it is not uncommon to find more than half of the enzymatic activity abolished with the standard method (*72*). Glucose oxidase seems to be less affected by oxidation. Moreover, some amino acids, such as methionine, may be oxidized and become more hydrophilic, thereby altering the conformational stability of the enzyme. Excessive oxidation also favours the formation of large polymers.

To eliminate these problems to some degree and to simplify the procedure, we described a new method (*58*, *89*):

(i) dissolve 5 mg of peroxidase in 0.45 ml of a 0.1 mol/l of sodium bicarbonate solution and add, while mixing, 0.05 ml of a 0.05 mol/l sodium *m*-periodate solution (in distilled water). Close the tube and leave for 2–3 hours in the dark;

(ii) close the tip of a Pasteur pipette by flaming, introduce a glasswool filter to the constriction, and add subsequently (while mixing) 1.5 ml of IgG (10 mg/ml in 0.1 mol/l of carbonate buffer, pH 9.3) and the activated peroxidase solution;

(iii) add immediately dry Sephadex G-25 (one sixth of the combined weight of the solutions, i.e., 0.33 g). The beads will swell immediately; concentrate the protein solution about two-fold, and consume the excess periodate inside the beads;

(iv) after 2–3 hours, the conjugate is eluted from the Sephadex (after breaking the tip of the pipette) by adding carbonate buffer (0.1 mol/l, pH 9.3);

(v) the conjugate is stabilized by adding, at 30-min intervals, 1/20 and 3/20 volumes of sodium borohydride (5 mg/ml) solutions, each time freshly prepared.

This procedure may be directely adapted to glucose oxidase by taking the relative molecular mass of the latter into consideration. The peroxidase conjugate prepared can be purified conveniently by precipitation of the IgG and IgG-peroxidase by adding an equal volume of saturated ammonium sulfate (for most of the mammalian IgG, affinity purification protein A-Sepharose may be used instead). Secondly, IgG is separated from IgG-peroxidase by Con A-Sepharose chromatography (*58*, *88*). The sample is passed over a Con A-Sepharose column equilibrated with PBS, and the conjugate retained by Con A is eluted subsequently by adding 0.01 mol/l of methyl-*D*-mannopyranoxide in PBS. This method is, however, not applicable in cases where the carbohydrate moiety has been overoxidized. Moreover, a small fraction of the IgG (about 5%) has an affinity for Con A. It is, therefore, recommended to pass the IgG over a Con A column prior to labelling in order to remove those parts having an affinity for Con A from the pool.

Antibody chimera by the glutaraldehyde method. This method (*77*) is simple and convenient when monoclonal antibodies to peroxidase are available. The antibodies (0.1 mg IgG) are mixed with 0.8 mg anti-peroxidase IgG in 1 ml of 0.1 mol/l phosphate buffer, pH 6.8, containing 0.1% glutaraldehyde, for a period of 2 hours at room temperature. Subsequently , 0.05 ml of 2 mol/l of glycine is added and the mixture is incubated for another 2 hours, followed by dialysis overnight against PBS. Precipitated protein is removed by centrifugation and the supernatant is used directly in the EIA. The antibody-chimera, at about 0.5 mg antibody/l, is incubated with a 10-fold molar excess of peroxidase for 1 hour at room temperature and diluted 2–3-fold for use.

Glutaraldehyde method for conjugation of alkaline phosphatase to IgG. All buffers should be prepared with at least double-distilled, sterile water. The following procedure is according to Avrameas et al. (*5*):

(i) dialyse extensively 2 ml of a solution containing 10 mg alkaline phosphatase and 5 mg IgG (or 2.5 mg Fab), against 0.1 mol/l phosphate buffer, pH 6.8;

(ii) add dropwise 0.05 ml of a 1% glutaraldehyde solution and incubate for 2–3 hours;

(iii) block the reactive sites by adding an excess (1/20 volume, 1 mol/l) of *L*-lysine and dialyse extensively.

Conjugation of β-galactosidase to IgG or Fab by N, N′-*o-phenylenedimaleimide*. This method requires the presence of sulfhydryl groups in both macromolecules to be conjugated. These thiol groups should be present in β-galactosidase (theoretically 40 per molecule, but in practice considerably less may be found). In IgG some of the interchain disulfide bridges can be reduced to generate the needed thiol groups. The following method is adapted from Hamaguchi et al. (*37*):

(i) dialyse 6 mg IgG or $F(ab')_2$ in 0.9 ml against 0.1 mol/l of sodium acetate buffer, pH 5.0;

(ii) reduce the disulfide bonds by adding slowly 0.1 ml of 0.1 mol/l 2-mercaptoethylamine and incubate for 90 min at 37 °C under nitrogen;

(iii) remove the reducing agent by filtration through Sephadex G-25 (30 ml);

(iv) collect the proteins in a saturated solution of *N*, *N′*-*o*-phenylenedimaleimide in the same buffer, and incubate for 20 min at 30 °C;

(v) remove the cross-linking agent on Sephadex G-25 and concentrate the maleimide-derived antibody solution to 0.3 ml;

(vi) adjust the pH to 6.5 with 0.25 mol/l sodium phosphate buffer, pH 7.5;

(vii) add successively 20 μl of *N*-ethylmaleimide-BSA, 1 μl of 1 mol/l magnesium chloride, and 0.1 ml of β-galactosidase (5 mg/ml), and incubate for 16 hours at 4 °C;

(viii) bring the volume of the sample with 0.01 mol/l sodium phosphate buffer to 1 ml, pH 7.0, containing 0.1 mol/l $CaCl_2$, 1 mmol/l $MgCl_2$, 0.1% BSA, and 0.1% sodium azide;

(ix) separate from unconjugated IgG or Fab′ by chromatography on a 80 ml Sepharose 6B column.

Conjugation of β-galactosidase to antibody or antigen using m-*maleimidobenzoyl*-N-*hydroxysuccinimide ester (MBS)*. MBS has the advantage over *N*, *N′*-*o*-phenylenedimaleimide in that only one of two macromolecules (i.e., β-galactosidase) needs to contain free thiol groups. The *N*-hydroxysuccinimide group of this cross-linker reacts directly to amino groups. The method presented here will conjugate virtually all β-galactosidase (*75*):

(i) dialyse IgG (1 mg/ml) against 0.1 mol/l phosphate buffer, pH 7.0, containing 0.05 mol/l sodium chloride;

(ii) add 10 μl of dioxan, containing 20 mg MBS/ml, to each ml of antibody solution, mix, and incubate for 1 hour;

(iii) remove excess of reagent by passage through a Sephadex G-25 column equilibrated with the phosphate buffer supplemented with 10 mmol/l $MgCl_2$;

(iv) collect the antibody-containing fractions and mix immediately with β-galactosidase (amount equal to the weight of IgG) and incubate for 1 hour at 30 °C;

(v) stop the reaction by adding 2-mercaptoethanol to a concentration of 0.01 mol/l and purify as described in the previous section.

N-hydroxysuccinimide ester of 4-(N-maleimidomethyl) cyclohexane-1-carboxylic acid (CHM-NHS) for efficient conjugation. CHM-NHS is very useful for the conjugation of peroxidase, alkaline phosphatase and glucose oxidase, but less for β-galactosidase (*47*). As with all maleimide procedures, the use of azide should be avoided since it decomposes maleimide. The method presented here is for peroxidase. For glucose oxidase, the modifications are that 5 times less CHM-NHS is used, and that 2 mg of thiolated IgG is used per mg of enzyme. For alkaline phosphatase, a different buffer should be used to maintain an active enzyme (0.05 mol/l sodium borate, pH 7.6, containing 1 mmol/l $MgCl_2$ and 0.1 mol/l $ZnCl_2$):

(i) dissolve 2 mg peroxidase in 0.3 ml of 0.1 mol/l sodium phosphate buffer, pH 9.0, and add 20 μl *N, N'*-dimethylformamide containing 1.6 mg CHM-NHS;

(ii) incubate for 1 hour at 30 °C;

(iii) remove the precipitate by centrifugation and pass the enzyme through Sephadex G-25 column equilibrated with the phosphate buffer to remove free cross-linker;

(iv) concentrate the enzyme in the cold and add 4 mg thiolated or reduced IgG (as described above) (final concentration should be 0.05 and 0.15 mmol/l, respectively);

(v) incubate for 20 hours at 4 °C and add 1 mmol/l of 2-mercaptoethylamine to block the remaining maleimide groups;

(vi) purify the conjugate by the method described above.

THE SOLID PHASE

Plastic is the most generally used solid-phase material in EIA. Polystyrene causes less background staining than polyvinyl and is, therefore, very popular. The nature of the interaction of the antigens or antibodies with the plastic is not clear. However, hydrophobic binding is most important. Once the first layer of immunoreactants is immobilized, a non-ionic detergent (Tween 20, Triton X-100) or an excess of "inert" protein (albumin or gelatine) is included in the solutions to prevent nonspecific adsorption.

The most popular formats of the plastics are microplates with 12 × 8 wells (round or flat-bottom), strips of single rows of wells, and rows of cuvettes which, according to its manufacturer (Gibco), show less than 3% variation. For the microplates, "edge" effects are often apparent. However, to forego their use would mean a loss of almost 40% of material. Much of this edge effect may be due to the poor conduction of heat by polystyrene (*74*).

Plastics have, however, some important limitations: (i) they use a large amount of immunoreactant, (ii) the avidity for large antigens decreases quite often upon immobilization, and (iii) the antibody-antigen interactions are quite slow. Hermann et al. (*43*) observed that up to 68% of noncovalently adsorbed viral antigens may be desorbed from the plastic during the test. This desorption can also be influenced strongly by the serum used (*20*). Large differences can be obtained with different batches of plates; some bind albumin poorly whereas others adsorb this protein well (*50*). The latter are better suited for mixtures of antigens.

Nitrocellulose is still not very widely used but is very promising since it can be used (i) with very small samples (less than 1 μl) and (ii) with antigens solubilized with detergents. Moreover, nitrocellulose binds close to 100% of most antigens, whereas plastics bind usually less than 10%.

Guidelines for solid-phase immobilization

Buffers generally have little or no effect on the immobilization of proteins. Carbonate (0.05 mol/l, pH 9.6), Tris-saline (0.01 mol/l Tris-HCl, pH 8.5, with 0.1 mol/l NaCl) and PBS (0.01 mol/l phosphate buffer, pH 7.2, with 0.1 mol/l NaCl) are widely used. Barlough et al. (*6*) observed, however, that in EIA for anti-coronavirus antibodies the widely used carbonate buffer was less suitable, and high background staining was obtained. The use of PBS, 0.9% NaCl, or deionized water for coating of coronavirus antigens produced better results.

Incubation overnight at 4 °C is the most common method, but may be shortened for most purposes

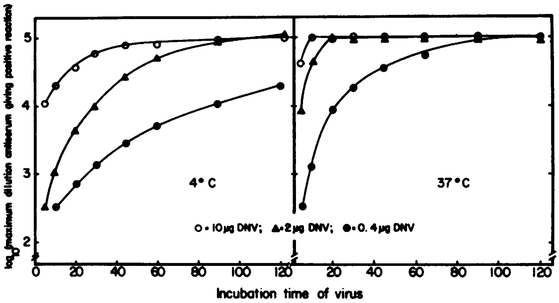

Fig. 1. Secondary plots of the results obtained with different conditions of sensitization of the polystyrene plates with densonucleosis virus (DNV). Three different concentrations of virus (0.4, 2, or 10 μg) were incubated for various periods of time at either 4 °C or 37 °C. The dilution which gave a response of 0.15 OD/30 minutes was determined on a primary plot and plotted against the time on the secondary plot.

(Fig. 1). Densonucleosis virus (a parvovirus) required very short incubation periods at 37 °C, about 20 min (*88*), whereas IgG is optimally coated during 1 h at 37 °C. The optimal concentration is, depending on the antigen or antibody, between 1 and 10 μg/ml. If complete antiserum is used, an optimum is obtained at a serum dilution of 1:10 000.

Partial denaturation of the proteins to be coated may increase the sensitivity of the EIA system (*13*), probably because of increased hydrophobic interaction between the plastic and the protein. IgG is partially denatured at the Fc site by dissolving the IgG in 0.05 mol/l glycine-HCl buffer, pH 2.5, containing 0.1 mol/l NaCl, incubating for 10 min at room temperature, and neutralizing with 0.5 mol/l Tris.

For lipid antigens or viral envelopes the inclusion of sodium deoxycholate (1 mg/ml) was found advantageous and sometimes essential.

Nitrocellulose can act as the solid phase by the application of minute drops (e.g., 0.5 μl) containing about 100 pg of antigen or antibody and subsequently drying. The sensitivity of this system is much higher than that of the assays using microtitration plates and titres of 10^7 for antisera are common. This method is, however, limited to visual inspection, though reflectance densitometry is possible. Nitrocellulose can also be used for transferring proteins from polyacrylamide gels and subsequent EIH on the membrane.

ENZYME IMMUNOASSAY PROCEDURES

All EIA procedures can be divided into two stages: the immunological and the enzymatic. For each, a large number of different systems exist, and many different combinations can be made. In this review we will limit ourselves to the solid phase EIA, since with rare exceptions these are preferably used in the diagnosis of infectious diseases.

Immunological interactions in EIA

Two fundamentally different solid phase EIAs can be distinguished, the noncompetitive and the competitive assays. One of the immunoreactants (antigens, antibodies), or other binders (e.g., complement such as C1q receptors) is immobilized on the solid phase. Sometimes it may be necessary to perform a postcoating with an "inert" protein (e.g., gelatin) if nonspecific binding occurs.

Buffers used for dilution or washing generally contain 0.05% Tween or inert proteins (BSA, gelatine) to prevent nonspecific adsorbance to the solid phase. The most common buffers are PBS-T (PBS, pH 7.4, with 0.05% Tween-20), Tris-T (0.02 Tris-HCl, pH 7.4, with 0.15 mol/l NaCl, 5 mmol/l KCl and 0.05% Tween-20), or PBS-GM (PBS supplemented with 0.5% gelatin and 1 mmol/l $MgCl_2$). Tris-T is used in particular for alkaline phosphatase assays whereas PBS-GM is recommended for immunoassays using β-galactosidase. Positive and negative reference samples should be included in the tests. In the sandwich assay, tests for rheumatoid factors are essential.

The optimum incubation conditions should be established for each particular system. For example, incubation at 37 °C may be excellent for one system, but will give poor results with another owing to the nature of the antibody-antigen interaction. Generally, incubation for 2 hours is sufficient.

Noncompetitive EIA

Two situations can be distinguished: the detection of antibodies and of antigens (Fig. 2). The most employed EIA for the detection of antibodies is the indirect method, in which the antigen is immobilized on the solid phase. This antigen will capture antibodies from the test serum which can then be detected by enzyme-labelled anti-immunoglobulin antibodies. Preliminary tests should be performed to determine at which concentration the added antigens or conjugates are nonlimiting. If microplate readers are used, some investigators prefer to use the conjugate at a lower concentration as indicated below (see section on quality control and standardization).

An increase in detectability can be achieved by further amplification, e.g., by adding a step such as the use of BSA-antibody conjugates in the last step, followed by an amplification with anti-BSA antibodies conjugated with enzyme (*35*). Alternatively,

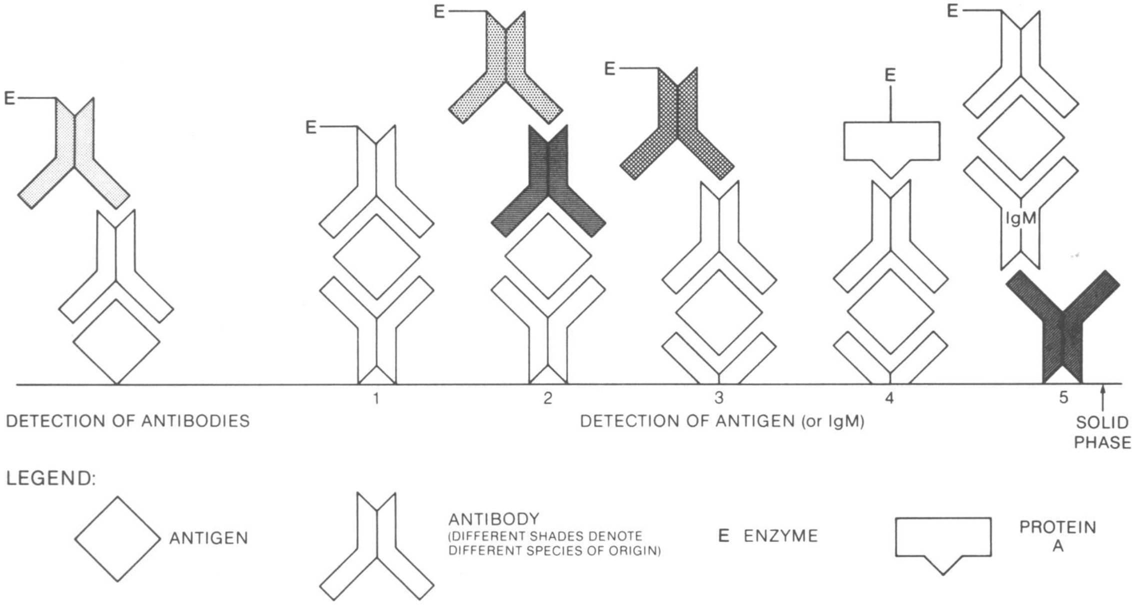
E
IgM
1
2
3
4
5
DETECTION OF ANTIBODIES
DETECTION OF ANTIGEN (or IgM)
SOLID PHASE
LEGEND:
ANTIGEN
ANTIBODY (DIFFERENT SHADES DENOTE DIFFERENT SPECIES OF ORIGIN)
E ENZYME
PROTEIN A

Fig. 2. Variations of solid-phase EIA used in immunodiagnosis. The labelled antibody in this diagram may consist of several layers of immunoreactants (bridge, double bridge, etc.). For the detection of antibodies, antigen is immobilized to serve as a trapping reactant whereas for the detection of antigens the antibody is immobilized. Most common is the direct sandwich method (1); if the indirect sandwich method is used, antibodies should be obtained from two different species (2), or the F(ab′)$_2$ fragments are immobilized so that the conjugate can only recognize the Fc fragment of the second antibody layer (3). The use of enzyme-conjugated protein A as a universal detection agent is particularly useful (4). In the class capture technique (5), the macromolecule to be detected is also in the second layer (5). According to the design, a higher detectability or higher specificity is obtained.

enzyme-coupled protein A may be employed in the last assay step, followed by enzyme-coupled anti-protein A antibodies (*44*).

The indirect sandwich methods, which give a wider specificity, have the disadvantage that antisera from two different species are required. Barbara & Clark (*97*) circumvented this problem elegantly. Since the anti-immunoglobulin antibodies recognize the Fc portion of the antibody, they immobilized Fab′ fragments on the solid phase, followed by the test sample, the antiserum (same as that from which Fab′ was prepared), and the conjugated anti-immunoglobulin antibodies. Alternatively, it is possible to use conjugated protein A. This versatile method has a detectability at least as good as the method using two different antisera, has lower background staining, and compares well with respect to the broadening of specificity desired. The various designs of sandwich assays are represented in Fig. 2 (series 1-4).

General bridge methods can also be employed to amplify the response such as the use of immunologically linked enzyme (*11*), the avidin-biotin complex (*66*), or lectin-based bridging (*34*). Incubation conditions are similar to those for antigen-antibody interaction with the exception of the avidin-biotin interaction for which there is a pronounced optimum of about 20 min at room temperature (*49*). The avidin-biotin method may increase the detectability 20-100-fold, but has also some limitations: avidin is a very basic glycoprotein and thus may be bound non-specifically (*40*), and test samples may contain one or more biotin-containing enzymes.

As an example of the avidin-biotin protocols, the following procedures (all using PBS-T) may serve as a guideline (in these assays the antigen is coated to the solid phase by conventional methods, followed by an incubation with the antiserum to be tested, and an incubation with biotin-conjugated anti-IgG antibodies):

(i) Enzyme-labelled avidin-biotin method (*33*). Avidin conjugated with enzyme, at a concentration of about 0.15 mg/ml is added to the biotinylated antibody immobilized on the solid phase and incubated for 2 hours at room temperature. The plates are washed well and substrate is added.

(ii) Bridged avidin-biotin method (*33*). Avidin at a concentration of 10 μg/ml is added to the immobilized biotinylated antibody and incubated for 15 min at room temperature. The plates are washed extensively and biotinylated enzyme at 1 μg/ml is added, followed by incubation for 1 hour at room temperature. After washing, the substrate is added.

(iii) The avidin-biotin complex (ABC) procedure (*46*). In this method, avidin and biotinylated peroxidase are mixed 20–200 min prior to use in ELISA, so that the end concentration becomes 5 μg avidin and 2 μg biotin-peroxidase per ml (performed at room temperature). This mixture is added to the immobilized biotin-antibody and incubated for 20 min at room temperature. After washing extensively, the substrate is added.

Competitive EIA

The most frequent competitive enzyme immunoassays are those in which antibodies are immobilized on the solid phase. The antigen to be tested is then applied in the presence of enzyme-labelled antigen. The more antigen is present in the test sample, the less enzyme (activity) will be bound to the solid phase. Two approaches may be taken: (i) simultaneous competition in which antigen and enzyme-labelled antigen are added together; or (ii) sequential saturation, in which the antigen is added first, followed by an incubation with enzyme-labelled antigen. The decrease in enzyme activity is larger in the second approach, but the specificity is lower (*58*).

Alternatively, enzyme-labelled antibody can be prevented from binding to the antigen immobilized on the solid phase by the presence of antigen in the test sample, which is added to the plate simultaneously with the enzyme-antibody complex (*2*), or sequentially (*87*). In the latter case, the test antigen is first incubated with the labelled antibody and the mixture is then added to the immobilized antigen. This method has been shown to be useful for the determination of the specificity of monoclonal antibodies (*26*).

Determination of enzyme activity in solid phase assays

The solid phase will influence the kinetic parameters of the immobilized enzyme. For example, the zeta-potential of the solid phase may cause attraction or repulsion of the substrate or product. Diffusion to the thin layer (1 μm) close to the solid phase is generally limited. It is therefore recommended that optimal conditions should be established in preliminary tests for each batch of solid phase. In our experience, optimum conditions are generally very different for the solid phase from those for fluid phase enzyme systems.

Another factor that should be taken into account is the length of the incubation period. Most quantitative tests are designed so that the build-up of coloured product is measured after a fixed period (e.g., 30 min). Systems in which a high initial activity is obtained, do not necessary yield most products after 30 min. For example, peroxidase will, with high concentrations of peroxide, show a high initial activity, but since the substrate is also a powerful inhibitor, less product is formed after 30 min. Gallati & Brodbeck (*27*) observed that for incubation periods of 5 min 10 mmol/l peroxide was optimal, whereas for 30 and 60 min this was, respectively, 5 and 2.5 mmol/l.

The chromogenic hydrogen donors of choice for peroxidase depend on the system, e.g., 4′, 4′-diaminobenzidine in immunohistochemistry, 4-chloro-1-naphthol in immunoblotting, and the most frequent donors used in EIA are *o*-phenylenediamine (OPD) and 5-aminosalicylic acid (5-AS), as well as 2,2′-azino-di (3-ethylbenzothiazoline-6-sulfonate (A-BTS), *o*-dianisidine, *o*-tolidine, and the MBTH-DMAB pair (MBTH is 3-methyl-2- benzothiazolinone hydrazone; DMAB is 3-(dimethylamino) benzoic acid. The relative merits of these have been compared (*1*, *10*, *83*, *91*). Generally, 5-AS and OPD were found to be most satisfactory. None of these comparisons used the purified 5-AS, which in our experience has important advantages.

Ellens & Gielkens (*22*) reported a method to purify 5-AS from the commercially available crude preparations. The latter contain considerable amounts of impurities which are hardly soluble. However, the purified 5-AS is very soluble, and is white compared with the purple commercial product. About 5 g of 5-AS and 5 g of sodium bisulfite are dissolved in 550 ml of deionized water at 80 °C and maintained for about 10 min at this temperature; 2 g of activated charcoal are added and the suspension mixed for about 5 min. The hot solution is filtered and then cooled to 4 °C. The precipitate is washed twice with 5 ml water at 4 °C and subsequently dried in the dark.

The purified H-donor is redissolved at a concentration of 1 g/l in 10 mmol/l phosphate buffer, pH 6.0, containing 0.1 mmol/l EDTA. This stock solution can be stored frozen. Upon thawing, a precipitate is present in the test-tube which is readily redissolved under a hot tap when the solution is brought to room temperature.

ABTS is prepared at a concentration of 0.4 mg/ml in 0.1 mol/l phosphate-citrate buffer, pH 4.0. OPD was originally used at the same concentration but Caillaud & du Pasquier (*12*) demonstrated that higher concentrations (2 mg/ml) gave much better results. The buffer used for OPD is 0.1 mol/l citric acid adjusted to pH 5.0 with sodium hydroxide. The OPD solution should be shielded from light (e.g., the container may be wrapped in foil); *o*-tolidine is prepared by dissolving 21.2 mg in 1 ml dimethylformamide, and this mixture is added to 100 ml of 0.1 mol/l citric acetate buffer, pH 3.7. The peroxide concentrations in these chromogenic solutions should be 0.003% for 5-AS, 0.009% for *o*-tolidine, and 0.02% for OPD.

The reaction can be stopped after a predetermined period by adding 0.5 volume of 4 mol/l sulfuric acid (in the case of OPD), 0.1 volume of 1 mol/l hydrochloric acid for MBTH/DMAB, and 0.1% sodium dodecyl sulfate for ABTS.

Glucose oxidase, which produces H_2O_2 from glucose can be detected indirectly by adding 0.28 mol/l glucose, 1 mg/ml ABTS, and 25 μg/ml peroxidase in 0.067 mol/l phosphate buffer.

β-galactosidase is detected colorimetrically with a substrate solution containing 70 mg *o*-nitrophenyl-*B*-*D*-galactopyranoside in 100 ml of 0.1 mol/l potassium phosphate buffer (pH 7.0), supplemented with 1 mmol/l $MgCl_2$ and 0.01 mol/l 2-mercaptoethanyl.

Alkaline phosphatase can be detected with a solution containing 97 ml diethanolamine (adjusted to pH 9.8 by the addition of HCl), 0.01% $MgCl_2$ and 0.1% *p*-nitrophenylphosphate.

RECORDING AND INTERPRETATION OF RESULTS OBTAINED WITH EIA

Dose-response curves for antigen or antibody usually have a sigmoidal form on which a level has to be set to discriminate between positive and negative results. Often a choice has to be made between accepting the relatively more false positives and the more false negatives (type I and type II errors, respectively), since avoiding both is virtually impossible. A compromise frequently chosen is to take the mean of the negative serum control plus two or three standard deviations of the mean (*15*, *79*), or to include a reference serum as an internal standard in each test to establish the cut-off value (*90*).

Some methods are based on visual inspection and yield only semi-quantitative or qualitative results. The titration method (endpoint determination) is not very precise unless the point at which the dose-response curve intersects the cut-off level is determined with a spectrophotometer. Moreover, this would yield a continuous scale. Titration procedures have the major advantage that the titre is directly indicative of the antibody activity. A problem is that the discrimination level cuts the dose-response curve in the tail-part of the sigmoidal curve and, consequently, low accuracy is obtained. Leinikki & Passila (*62*) tried to avoid this problem by measuring the antibody curves at highest sensitivities (i.e., in the straight part of the curve) and to compare these with a positive reference serum. The result is then expressed as the logarithm of the difference in dilution of the two sera at the highest sensitivities. This effective-dose (ED) method has improved reproducibility and the results are linearly proportional to antibody activity. This method assumes parallelism of the dose-response curves.

However, an important observation for antibody dose-response curves is that these curves are rarely parallel, owing to differences in concentration and affinity. Therefore, at one dilution one serum may indicate a higher activity, whereas at another dilution (at the other side of the cross-over), the inverse situation is encountered. Another problem is the occurrence of antibodies in different immunoglobulin classes which could cause a prozone effect (*18*). Moreover, the affinity of the antibodies trapped by the immobilized antigen tends to change with the dilution. Lehtonen & Eerola (*61*) observed that at low dilutions the antibodies of high affinity are bound, whereas at high dilutions the antibodies with a wider range of affinities are bound. This problem has not yet been resolved.

These important differences should be taken into account for the different modes of expression of the results. It is also necessary to stress the problems associated with the determination of the discrimination level to distinguish between positive and negative samples. Intra- and interassay precision and accuracy of the results need to be assessed. To improve these parameters, different methods have been adopted to linearize the sigmoidal dose-response curves and computer programs have been designed for rapid processing of EIA data (*98*).

The cut-off value

Most assay methods have a built-in discrimination level for positive and negative results. This level may be set at 0.15 or 0.20 adsorbance (*36*), or at two or three times the mean (*67*), or at the mean plus two or three standard deviations (SD) (*79*). It should be stressed that the SD estimation is subject to error as well, and depends on the number of responses from which the standard deviations were calculated.

A compromise is generally necessary in setting the cut-off level since, irrespective of its value, false positives (type I errors) or false negatives (type II errors) are encountered. Moving the level to higher values will minimize the false positives at the cost of more false negatives and vice versa. It is also possible to define the responses in this intermediate area as "doubtful" (*39*), e.g., between the mean of negatives and the mean plus four standard deviations.

General experimental approaches have been proposed to reduce the expenditure and time involved in establishing the cut-off value. Van Loon & van der Veen (*90*) established the mean and standard deviation of a large group of normal sera and, in parallel, tested the reference sera. It was then observed that the mean plus three standard deviations equalled 40% of the adsorbance of a reference serum. This reference serum was included in subsequent tests as an internal standard to establish the cut-off value. Cremer et al. (*15*) included both standardized positive and negative sera.

Methods of expressing serological results in enzyme immunoassays

Semi-quantitative tests. Visual inspection has been used frequently for rapid screening by enzyme immunoassays on microtitration plate or on other solid phases, such as nitrocellulose (immunodotting). The detectability is in the same order of magnitude as adsorptiometric methods. However, it is quite subjective and difficult to distinguish positive from negative responses in the "doubtful" range. Moreover, such titration methods yield a discontinuous scale of results and require a series of dilutions. Nevertheless, the titre obtained is proportional to the antibody activity. A more precise endpoint is obtained by determining the optical density at each dilution and establishing at which dilution the dose-response curve intersects the cut-off level.

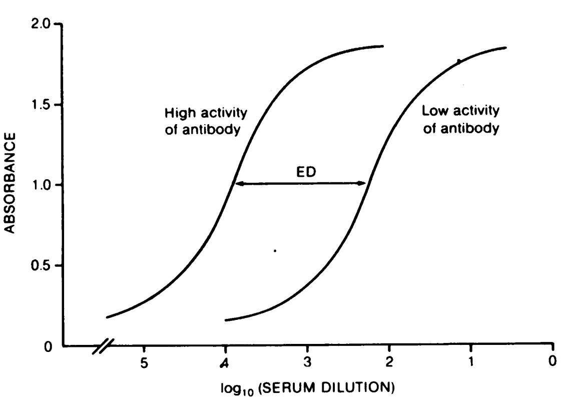

Fig. 3. The determination of the effective dose (ED). The optical density in wells with increasing concentrations of the serum to be tested and of a reference serum are compared at the highest sensitivity (i.e., at the steepest section of the dose-response curve). The difference in dilution of the sera to obtain this same optical density, is expressed in Brigg's logs.

Effective dose method. The effective dose method, proposed by Leinikki & Passila (*62*), compares the dose-response curves obtained by a dilution series of the test serum and a dilution series of the positive reference serum. The difference between the dilutions at the steepest point of the dose-response curves of two sera is expressed in Brigg's logs (Fig. 3).

Reproducibility of this method is better than that of the titration method since in the latter the result is established in an area of low precision (tail of the sigmoid curve). The effective dose is also proportional to antibody activity. Disadvantages of the effective dose method are the time-consuming and expensive way of activity measurement (similar to the titration method) and the uncommon way of expressing the result (e.g., effective dose = −0.49).

The absorbance method. The use of chromogenic substrates leads almost automatically to the use of absorbance methods. Few or only one suitable dilution needs to be made to obtain the result. However, there is no direct relation of antibody activity to the optical density, and the latter is not proportional to the antibody activity. De Savigny & Voller (*18*) pointed out the limited understanding of absorbance values by clinicians. An advantage, in addition to the

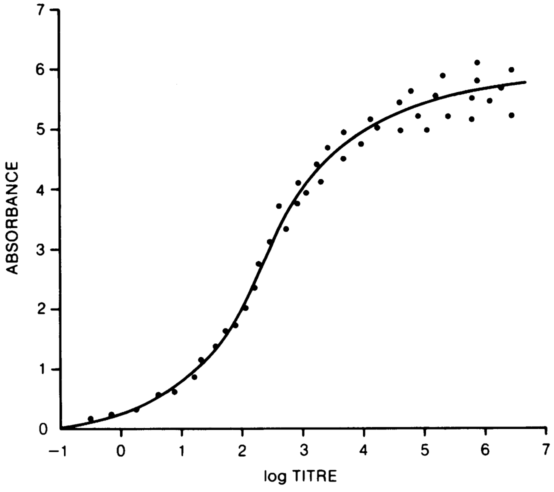

Fig. 4. Standard curve obtained from a large number of sera (negative, weakly positive, strongly positive) by plotting the absorbance at a fixed dilution (e.g., 1:1000) against the titre of that serum. Variation increases significantly with the titre. The sigmoid curve may be linearized by several methods, making it easier to estimate the titre of serum if a certain optical density is obtained. The standard units obtained are directly proportional to antibody activity.

single-dilution requirement, is that the scale is continuous, though it may not be reliable outside 0.2–0.8 units.

Standard unit curves. In this method the absorbance values are transformed to standard units, which yield a continuous scale and are proportional to the antibody activity. Moreover, single dilutions of each serum are needed, if it is assumed that dose-response curves for the various sera are parallel. This method is illustrated in Fig. 4.

The standard curve is constructed from plotting the absorbance obtained from sera at a single dilution (e.g., 1:1000) against their respective titres. The test samples in subsequent experiments are measured for activity at the same dilution (internal standards from the original curve can be included) and the titre is obtained directly from the standard curve. Malvano et al. (*67*) used a similar approach, with the exception that instead of a battery of different sera, one positive serum, calibrated against a WHO standard serum and diluted at varying degrees with a negative serum, was used to construct the standard unit curve.

With these approaches it is necessary to include internal standards for between-run, between-laboratory, and between-method normalization.

Ratio methods. Different ratio methods are used, in which the test sample absorbance is expressed in terms of a reference serum. The P/N (positive/negative serum absorbance) (*65*) is frequently employed. A P/N greater than 2 or 3 is considered positive. This method is simple and easily understood, and not time-consuming or expensive. However, reproducibility is poor and the ratios (dependent on a particular negative reference serum) are not directly proportional to the antibody activity.

Sedgwick et al. (*84*) devised a ratio method based on the areas under the dose-response curves (at a few dilutions). This method does not assume that the dose-response curves are parallel. The ratios obtained are proportional to the antibody activity and have better reproducibility. The method is however, more time-consuming and expensive.

Multiple of normal activity (MONA). Felgner (*24*) considered the lower half of the sigmoidal dose-response curve, and calculated the parabola exponent n from this part (Fig. 5) with the formula:

$$\left(\frac{A_{\mathrm{ser}}}{A_{\mathrm{ser\ dil.}}}\right)^n = \text{dilution factor}$$

on which A_{ser} is the absorbance at fixed dilution and $A_{\mathrm{ser\ dil.}}$ the absorbance at further dilutions. This A_{ser} should be below the point of inflexion on the dose-response curve.

The absorbance of the test serum can be expressed in MONA by the following formula:

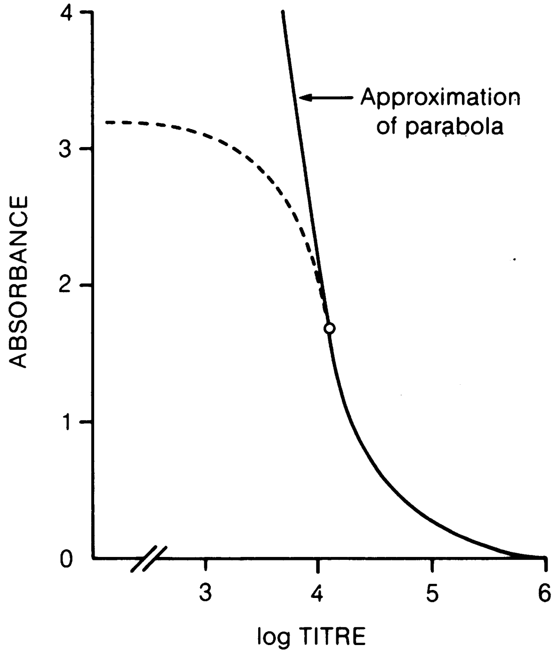

Fig. 5. The determination of MONA (mulitple of normal activity) values according to Felgner (*24*). The sigmoid curve is considered until its midpoint (indicated by o), and the lower part of this dose- response curve can be approximated by a parabola, by calculation of the parabola exponent taking several serum dilutions. In this approach the levelling off of the dose-response curve at high antibody concentrations is artificially avoided.

$$\log_{10} \mathrm{MONA}_{\mathrm{test}} = n\,(\log_{10} A_{\mathrm{test}} - \log_{10} A_{\mathrm{neg.ser.}})$$

The parabola exponent may change from experiment to experiment, but the MONA will be rather stable.

This method has several advantages, such as (i) a single dilution and improved sensitivity, (ii) it is proportional to the antibody activity, and (iii) it compares the result directly with the negative serum. It is assumed in this approach that the dose-response curves are parallel and the determination of n may be greatly influenced by nonparallelism.

The assumption that nonspecific binding is constant over the complete range is not valid since it ignores the law of mass action. Negative sera should therefore be tested at the same dilutions as the test and positive sera.

Linearization of dose-response curves. Transformations to obtain linear dose-response curves are of little use in solid-phase EIA since the compression of errors leads only to an apparent improvement of data. On the other hand, care should be taken to avoid linear interpretation or to assume linearity.

Linearization is particularly popular in determination of hapten concentration. Several mathematical models have been developed (see Oellerich et al. (*73*)):

Four-parameter log-logit model:

$$R = R_0 + K_c \cdot \frac{1}{1 + \exp\left[-(a + b \ln C)\right]}$$

Five-parameter logit:

$$R = R_0 + K_c \cdot \frac{1}{1 + \exp\left[-(a + b \ln C + cC)\right]}$$

Five-parameter exponential:

$$R = R_0 + K \ \exp\left[a \ln C + b(\ln C)^2 + c(\ln C)^3\right]$$

Five parameter polynomial:

$$\ln C = a + b.\ \frac{(R - R_0)}{100} + c.\ \frac{(R - R_0)^2}{100} + d.\ \frac{(R - R_0)^3}{100}$$

Spline approximation:

$$C = a_i + b_i\,(R - R_i) + c_i\,(R - R_i)^2 + d_i\,(R - R_i)^3$$

where R is the rate of absorbance (R_0 is the predicted rate at zero concentration); K_c is the predicted difference between the maximum and minimum response (at infinite and zero doses, respectively); C is the concentration of the standards; and a, b, c, and d are various parameters to account for non-linearity.

Oellerich et al. (*73*) concluded that the four-parameter log-logit model is the most practical. The logit is defined (*7*) as ln (y/ 1-y) where y is a measurable response value.

The four-parameter log-logit model is analogous to the Hill equation (*3*) in enzyme kinetics, though some thermodynamic restrictions should be made (*17*). In commercial kits, the K_c given may often have to be changed for the particular laboratory conditions (*19*), otherwise the curves may not be straight. The slope is often low (about 0.5–0.8 (*80*)) so that a wide range of concentrations can be detected, but results in low sensitivity. Linearization generally leads to an artificial compression of errors at either end of the curve.

QUALITY CONTROL AND STANDARDIZATION

Quality control includes internal quality control (within laboratories) and external quality assessment. Internal control serves to ensure reproducibility of the assays, whereas external assessment is concerned with the validity of the experimental approach and the elimination of bias.

McLaren et al. (*69*) have compiled the major sources of errors in solid phase EIA, according to the sources responsible for low assay detectability, poor specificity, or low precision. Most of these possible errors have been discussed previously.

A scrupulous daily quality control is required and can be achieved by the inclusion of internal positive and negative reference reagents. The means, standard deviation, and coefficient of variation may be plotted on quality control charts. Within-assay reproducibility (= repeatability) and between-assay rerpoducibility should be assessed. The repeatability should have a coefficient of variation of less than 5%, whereas for between-assay reproducibility this should be no more than 10%.

Peroxidase conjugates (absorbance ratio of 0.3–0.4 at 403/278 nm) should, at a concentration of 5 ng/ml in a substrate solution (40 mg OPD and 40 μl of 30% H_2O_2 in 100 ml at 20 °C), yield an absorbance at 492 nm of 1 after 15 min and 1.5 after 30 min if acidified with 1/8 volume of 1.5 mol/l HCl, or of 0.5 and 0.75 at 445 nm without acidification. These conjugates should be tested from time to time.

The working titre of the conjugate should be determined in preliminary tests. In tests that rely on visual assessment, an excess of conjugate is used so that maximum discrimination between positive and negative is achieved. In cases where the optical densities are measured, e.g., by microplate readers, it may be advantageous to decrease the conjugate concentration to that giving 1.0 OD/30 min in wells where the antigen is coated in excess, since the efficiency of the photometers is maximal between 0.2 and 0.8 OD, and background staining will be reduced. In the subsequent tests, a positive reference serum should be included to ensure reproducibility or to provide a means of correction.

CONCLUSIONS

The methods and approaches described in this review were selected from a large body of protocols that were found to be particularly satisfactory in our hands. The author does not pretend to have presented an exhaustive review of enzyme immunoassays, but has given an outline of their applicability, the problems often encountered, and the approaches to solve these difficulties. Without doubt, this exciting field will continue to evolve rapidly and become even more important among the diagnostic procedures in the future.

Unfortunately, many of the current procedures for enzyme immunoassays are performed suboptimally. The reagents are often of poor quality owing to considerable inactivation during the preparation of the conjugates. For example, conjugation of peroxidase by the currently popular periodate method results generally in an inactivation of the enzyme by at least 50%. Commercially available "pure" peroxidase is very expensive and contains several isozymes which are much less active. Purification of crude enzyme extracts is simple, costs about 10 times less and yields enzyme with much higher activity. This review has, therefore, also dealt with the problems of reagent production.

A second group of pitfalls for enzyme immunoassays is found in the designs. For example, alkaline phosphatase-mediated immunoassays are performed almost without exception in PBS. Phosphate is a powerful competitive inhibitor of this enzyme, decreasing the sensitivity of the assay by about 50%. Cross-reactivity of antisera may also cause a marked increase in false-positive results. As shown by numerous reports, increase in detectability is often at the expense of specificity. For example, direct sandwich assays are more specific than the indirect assays, but the sensitivity is less.

Of particular interest are the methods used for the recording of results. The importance of the difference between antibody dose–response curves and antigen dose–response curves (parallelism of curves, cut-off values, false-positives/false-negatives, methods of recording) was also discussed. In the future, the possibility of interfacing computers with readers should lead to better decision-making. Combined with automation and strict standardization, these new approaches will make enzyme immunoassays of prime importance in diagnostic medicine and biological research.

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RÉSUMÉ

PROGRÈS DANS LA PRODUCTION DE RÉACTIFS, LA TECHNIQUE EXPÉRIMENTALE ET L'INTERPRÉTATION DES TITRAGES IMMUNOENZYMATIQUES

Bien que les épreuves immunoenzymatiques soient utilisées à très grande échelle pour la détection d'antigènes ou des anticorps correspondants, souvent leur application ne se fait pas de façon optimale. La préparation d'un conjugué anticorps-peroxydase de raifort par la technique au periodate se traduit en général par une perte d'au moins 50% de l'activité de l'enzyme. La peroxydase peut être purifiée à partir d'extraits bruts par chromatofocalisation et chromatographie sur échangeur d'ions, ce qui permet d'obtenir un matériel dont le coût ne représente que le dixième de celui des produits disponibles dans le commerce. Cette enzyme possède une activité spécifique très élevée et n'est que très faiblement contaminée par des isoenzymes activées. La rétention de l'activité enzymatique semble plus importante que la rétention de l'activité immunologique du conjugué, à condition que la diminution de l'activité immunologique córresponde seulement au blocage des paratopes et non à une perte de l'affinité des paratopes pour leurs antigènes respectifs. Différentes techniques de couplage ont été discutées, par exemple les pontages avidine-biotine et les liaisons chimiques et immunochimiques. La réaction de couplage suit la loi d'action de masse, la vitesse de formation du complexe étant proportionnelle au produit des concentrations des réactifs; par exemple, lorsque la concentration des macromolécules est multipliée par 5, le temps d'incubation nécessaire l'est par 25.

La distribution des molécules activées est assez irrégulière. On peut montrer qu'indépendamment du rapport molaire enzyme/IgG, seulement 37% des molécules d'IgG sont couplées à une molécule d'enzyme. Lorsque l'on augmente la concentration de l'enzyme, la quantité d'IgG libre diminue et le nombre d'IgG couplées à deux ou plusieurs molécules d'enzyme augmente. Il est important d'utiliser un rapport enzyme/IgG optimal, étant donné que la présence d'IgG libres diminue considérablement la sensibilité des épreuves immunoenzymatiques. La liaison de plusieurs molécules d'enzyme à une IgG conduit à la formation d'agrégats responsables de "bruits de fond" importants.

Plusieurs inconvénients des épreuves immunoenzymatiques tiennent à la technique expérimentale. La majorité des épreuves utilisant la phosphatase alcaline a lieu dans un tampon phosphate. Or, le phosphate est un inhibiteur de cette enzyme et, de ce fait, la sensibilité de l'épreuve décroît de 50%. Les réactions croisées des immunsérums causent aussi une augmentation importante des faux-positifs. L'importance des différences entre les courbes dose–réponse en anticorps et en antigènes (parallélisme des courbes, point de coupure, faux-positifs/faux négatifs, méthodes d'enregistrement des résultats) est évaluée. La possibilité d'automatiser et d'informatiser (avec lecture automatique) les épreuves et leur normalisation feront des titrages immunoenzymatiques un outil de premier choix dans le diagnostic médical et la recherche.

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